Recombinant human interferon- γ

Differences in glycosylation and proteolytic processing lead to heterogeneity in batch culture

Elisabeth M. A. CURLING, Paul M. HAYTER, Anthony J. BAINES, Alan T. BULL, Keith GULL,*
Philip G. STRANGE and Nigel JENKINS

Biological Laboratory, University of Kent, Canterbury CT2 7NJ, Kent, U.K.

Recombinant human interferon- γ (Hu-IFN- γ) produced by Chinese-hamster ovary (CHO) cells was analysed by immunoprecipitation and SDS/PAGE. Up to twelve molecular-mass variants were secreted by this cell line. Three variants were recovered after enzymic removal of all *N*-linked oligosaccharides or when glycosylation was inhibited by tunicamycin. The presence of three polypeptide forms rather than a single form suggested that proteolytic cleavage had occurred at two sites in both the glycosylated and non-glycosylated forms. Proteolytically cleaved IFN- γ was more prevalent in cell lysates than in the secreted glycoprotein. In common with naturally produced IFN- γ , both fully glycosylated IFN- γ (asparagine residues 28 and 100 occupied) and partially glycosylated product (thought to be substituted at position Asn²⁸) were secreted. This was deduced from the M_r of the glycosylated products and the relative amounts of sialic acid expressed by each variant. In contrast with naturally produced IFN- γ , non-glycosylated IFN- γ was also secreted by the transfected CHO cells. When the cells were grown in batch culture in serum-free medium under pH and dissolved-oxygen control, the proportion of non-glycosylated IFN- γ increased from 3 to 5% after 3 h, to 30% of the total IFN- γ present after 195 h. This change in the proportion of glycosylated protein produced was not seen when metabolically labelled IFN- γ was incubated for 96 h with cell-free supernatant from actively growing CHO cells. This implied that an alteration in intracellular glycosylation was occurring rather than a degradation of oligosaccharide side chains after secretion. The decrease in IFN- γ glycosylation was independent of the glucose concentration in the culture medium, but could be related to specific growth and IFN- γ production rates, as these declined steadily after 50 h of culture, in line with the increased production of non-glycosylated IFN- γ .

INTRODUCTION

Human interferon- γ (Hu-IFN- γ) is a lymphokine normally secreted by antigen-sensitized T-lymphocytes which stimulates MHC (major-histocompatibility-complex) Class II expression on cells present during an immune response and has potent anti-viral activity (reviewed by Trinchieri & Perussia, 1985). The gene has recently been cloned and expressed in *Escherichia coli* and monkey cells (Gray *et al.*, 1982), insect, yeast and CHO cells (Mutsaers *et al.*, 1986). Although each eukaryotic expression system produces a biologically active protein *in vitro*, the structure of oligosaccharides added to the IFN- γ polypeptide during the glycosylation process varies significantly with cell type. Utsumi *et al.* (1989) found that recombinant human IFN- β is glycosylated very differently when expressed in mouse epithelial, human lung carcinoma or CHO cells, which can lead to altered immunogenicity *in vivo* (reviewed by Konrad, 1989) owing to the generation of neutralizing antibodies (Steis *et al.*, 1988). Typically, recombinant proteins expressed in CHO cells most closely resemble the naturally occurring human proteins (Utsumi *et al.*, 1989). However, differences in the glycoprotein sialic acid content can occur in CHO cells that can lead to extensive charge differences (Kelker, 1983) and altered clearance rates *in vivo* (Ashwell & Hartford, 1982), owing to specific receptors in the liver that recognize asialoglycoproteins.

Both natural (Rindernecht *et al.*, 1984) and CHO-derived (Mutsaers *et al.*, 1986) Hu-IFN- γ express complex biantennary *N*-linked oligosaccharides attached to either the Asn¹⁰⁰ residue only, and/or to the Asn²⁸ residue, yielding a mixture of fully and

partially glycosylated proteins. Glycosylation of a protein is more complete towards the *N*-terminus, possibly because these sites are translated first (Hubbard & Ivatt, 1981). The deduced amino acid sequence of human IFN- γ predicts a mature polypeptide of 146 amino acids with a molecular mass of 17.1 kDa (Gray *et al.*, 1982). However, core polypeptide processing of both natural and recombinant IFN- γ has been reported (Rindernecht *et al.*, 1984; Arakawa *et al.*, 1986), directed to both the *N*- and to the *C*-terminal ends of the molecule, leading to the formation of up to six molecular-mass variants (Rindernecht *et al.*, 1984). An intact *N*-terminus is crucial to maintain anti-viral activity (Ichimori *et al.*, 1987; Hogrefe *et al.*, 1989); however, a complete *C*-terminus may (De La Maza *et al.*, 1987; Seelig *et al.*, 1988) or may not (Sakaguchi *et al.*, 1988) be necessary for full biological activity.

In order to study the fidelity of human IFN- γ production in CHO cells, the variants produced were analysed by [³⁵S]methionine labelling, pulse-chase analysis and the use of specific glycosidases. By using these data it was then possible to monitor, for the first time, the approximate proportion of each IFN- γ variant synthesized during laboratory-scale fermenter batch culture to assess the influence of the culture environment on IFN- γ heterogeneity.

EXPERIMENTAL

Materials

Cell line. The mutant cell line CHO-K1 DHFR⁻ which lacks

Abbreviations used: (Hu-)IFN- γ , (human) interferon- γ ; CHO, Chinese-hamster ovary; DHFR, dihydrofolate reductase; 3,4-DCI, 3,4-dichloroisocoumarin; 1,10-Phen, 1,10-phenanthroline; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; MEM, minimal essential medium; DTT, dithiothreitol.

* Present address: Department of Biochemistry and Molecular Biology, University of Manchester, Stopford Building, Oxford Road, Manchester, M13 9BT, U.K.

the gene for dihydrofolate reductase (DHFR) was co-transfected with the genes for human IFN- γ and DHFR, which allows the amplification of IFN- γ gene expression by the selection of methotrexate resistance (McCormick *et al.*, 1984). This line was provided by Wellcome Biotechnology Ltd. (Beckenham, Kent, U.K.) and was adapted for growth in serum-free medium (Hayter *et al.*, 1990). The DHFR/IFN- γ -gene copy number was amplified by increasing the methotrexate concentration in the medium to a maximum of 1 μ M.

Reagents. Proteinase inhibitors [3,4-dichloroisocoumarin (3,4-DCI), 1,10-phenanthroline (1,10-Phen), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and pepstatin A] were obtained from Sigma (Poole, Dorset, U.K.). The following mixture of inhibitors was used at all stages of immunoprecipitation to prevent proteolysis: 0.1 mM-1,10-Phen/0.1 mM-3,4-DCI/0.05 mM-E-64/pepstatin A (1 mg/l). Neuraminidase (from *Clostridium perfringens* type VI), tunicamycin (from *Streptomyces* sp.), 14 C-labelled low-molecular-mass markers, Protein A-Sepharose, methionine-deficient minimal essential medium (MEM) medium and AgNO₃ were obtained from Sigma. L-[35 S]Methionine was purchased from Amersham International (Amersham, Bucks., U.K.). *N*-glycanase (peptide: *N*-glycosidase F) was obtained from Genzyme Biochemicals (Maidstone, Kent, U.K.) and high-quality acrylamide/bisacrylamide mixture was from Bio-Rad (Watford, Herts., U.K.). For autoradiography, Kodak (Hemel Hempstead, Herts., U.K.) X-OMAT AR film (pre-flashed before use) was used. Monoclonal antibody 20D7 (IgG2a) raised against *E. coli*-derived recombinant IFN- γ was supplied by Celltech Ltd., (Slough, Berks., U.K.). Sterile 96-well plastic plates (Falcon) were obtained from Becton Dickinson (Oxford, U.K.). Powdered RPMI medium was obtained from Imperial Laboratories (Andover, Hants, U.K.) and glutamine supplement from Gibco (Uxbridge, Middx., U.K.).

Methods

Fermenter culture. Batch cultures of the CHO cell line producing IFN- γ were initiated in 2-litre stirred tank reactors (Bioengineering Ltd., Wald, Switzerland) controlled at pH 7.2 with a dissolved-O₂ concentration of 40% air saturation. The initial glucose concentration was set at either 10 mM or 20 mM (see the Results section), and the stirrer speed was 60 rev./min. Cultures were inoculated at a starting density 10⁵/ml. To make IFN- γ and metabolite measurements and to study the IFN variants produced, daily samples were taken, the cells removed by centrifugation and then the supernatants snap-frozen in liquid N₂ and stored at -70 °C before immunoprecipitation and analysis.

Production of 35 S-labelled IFN- γ . CHO cells producing human IFN- γ were seeded at 10⁵/well in 96-well plates and incubated overnight at 37 °C in an atmosphere of CO₂/air (1:19). The following day the cells were washed twice in methionine-free MEM supplemented with 2 mM-glutamine. The cells were then incubated with the same medium supplemented with [35 S]methionine (8 μ Ci/100 μ l) for 3 h at 37 °C, before harvesting the culture supernatant containing metabolically labelled IFN- γ . For pulse-chase analysis, [35 S]methionine was added for 90 min, then 'chased' with MEM medium supplemented with a 10-fold excess of unlabelled methionine (after three washes in this medium). The supernatants were then collected at the time intervals indicated and the cells solubilized with lysis buffer [50 mM-Tris/HCl (pH 8.2)/5 mM-EDTA/1% (v/v) Triton X-100/1% (w/v) deoxycholate supplemented with the proteinase-inhibitor mixture]. Both supernatants and cell lysates were immunoprecipitated as detailed below.

Immunoprecipitation of IFN- γ . Samples (100 μ l) of 35 S-labelled supernatant containing CHO-derived IFN- γ were diluted in 300 μ l of reaction buffer [0.05 M-Tris/HCl/5 mM-EDTA/5 mM-EGTA/0.5% (w/v) deoxycholate, pH 8.2]. For fermenter culture samples, 1 ml of unlabelled supernatant was diluted to 1.4 ml using the same reaction buffer. To pre-clear samples of any Protein A-Sepharose-binding components, 40 μ l of a 10% suspension of Protein A-Sepharose was added to each sample and incubated on a rotating wheel at 4 °C for 30 min and centrifuged. The pre-cleared supernatant (with the Sepharose removed) was then transferred to a new Eppendorf tube containing 10 μ g of purified anti-IFN- γ antibody (20D7) and incubated for 3 h on ice. A further 60 μ l of Protein A-Sepharose suspension was then added before rotating the samples at 4 °C for 60 min. Non-precipitated components were removed by sedimenting the Protein A-Sepharose by centrifugation (1 min at 6500 g). The pellet was washed twice in Wash Buffer 1 [50 mM-Tris/HCl/5 mM-EDTA/0.5% (v/v) Nonidet P40/0.5 M-NaCl/BSA (1 mg/ml), pH 8.0] and twice in Wash Buffer 2 (as above but without BSA and NaCl). The washed pellets were resuspended in 20 μ l of double-distilled water and 20 μ l of reducing sample buffer [62.5 mM-Tris/2.5% (w/v) SDS/0.1% (w/v) Bromophenol Blue/10% (w/v) sucrose/and 0.1 M-dithiothreitol (DTT)] and immediately boiled for 3 min to dissociate IFN- γ from the Sepharose beads. After pelleting the Sepharose, 5 μ l of each sample was taken for scintillation counting and 5–15 μ l samples were loaded on to 14%-(w/v)-polyacrylamide/SDS gels (Laemmli, 1970). Each lane received approximately the same amount of radioactivity. Gels were run at a constant current of 15 mA. Subsequently the gels were dried before autoradiography or silver-stained by the method of Johnson & Thorpe (1987). Autoradiographs were produced over 4–7 days on pre-flashed film, and both silver-stained gels and autoradiographs were quantified by conversion into absorbance units with a scanning densitometer (Bio-Rad model 1050) connected to an Amstrad 1640 microcomputer. From the scans obtained, the area under each peak (corresponding to each band) was integrated to allow the relative proportions of each IFN- γ variant to be estimated.

To ensure that the silver staining followed by scanning densitometry of the gel lanes was linear over the range of IFN- γ levels expected from fermenter cultures, two calibration curves were produced on separate days using purified IFN- γ (results not shown). The relationship between the number of integration units calculated for the IFN- γ bands and the amount of IFN- γ loaded was linear between 60 and 600 ng/track (approx. 3000–30000 i.u. of IFN- γ) on both curves. This range encompassed the amounts of IFN typically immunoprecipitated from 1 ml of culture supernatant.

Deglycosylation of IFN- γ . For complete deglycosylation, immunoprecipitated IFN- γ samples were boiled in 0.5% SDS and 0.1 M-DTT for 3 min, diluted with 0.55 M-sodium phosphate buffer (pH 8.6; 0.2 M final concn.) before Nonidet P40 was added [1.25% (v/v) final concn.]. Finally, *N*-glycanase was added to a final concentration of 10 units/ml before each sample was incubated at 37 °C for 16 h. Control *N*-glycanase-treated samples were prepared as described above, but with enzyme omitted. To remove sialic acid residues only, boiled immunoprecipitates were diluted in buffer containing 100 mM-sodium acetate (pH 5.5)/0.5% Nonidet P-40 and 0.5 M-NaCl before the addition of 0.1 unit of neuraminidase (10 units/ml) for 16 h at 37 °C. Control neuraminidase samples were treated as described above, but with enzyme omitted. To inhibit intracellular glycosylation, cells were pre-treated for 1 h before, and 3 h during, the methionine labelling of the synthesized IFN- γ with tunicamycin (10 μ g/ml) dissolved in culture medium.

Fig. 1. Analysis of IFN- γ variants produced by CHO cells

Immunoprecipitated samples of control or treated IFN- γ labelled with [35 S]methionine secreted by CHO cells were resolved by SDS/PAGE on a 14%-(w/v)-polyacrylamide gel and detected by autoradiography. Lanes 1 and 8, 14 C-labelled molecular-mass markers; lane 2, control (mock-treated) in the absence of *N*-glycanase; lane 3, *N*-glycanase treatment; lane 4, IFN- γ produced in the presence of tunicamycin; lane 5, control (mock-treated) in the absence of neuraminidase; lane 6, neuraminidase treatment; lane 7, control (no treatment).

RESULTS

Characterization of secreted IFN- γ

The secreted products of the human IFN- γ gene transcribed and translated by the CHO-cell line were analysed by SDS/PAGE (Fig. 1). Up to twelve different bands could be detected after immunoprecipitation with the monoclonal anti-IFN- γ antibody 20D7 (Fig. 1, lane 7, and Fig. 2a). Control immunoprecipitates from non-transfected (DHFR⁻) CHO cells, or where an irrelevant mouse antibody was used, produced no bands (results not shown), indicating that all twelve bands observed were forms of IFN- γ .

In order to discover which of the bands represented glycosylated IFN- γ , samples were completely deglycosylated using *N*-glycanase (Fig. 1, lane 3, and Fig. 2b) and compared with samples where incubations were identical except that the enzyme was absent (mock-treated; Fig. 1, lane 2, and Fig. 2a). *N*-Glycanase cleaves all high-mannose, hybrid, bi-, tri- and tetra-antennary complex oligosaccharides between the innermost *NN*-diacetylchitobiose and the asparagine residue. Complete removal of the oligosaccharide side chains decreased the number of bands seen to three, with molecular-mass values of 17, 16.2 and 15.2 kDa (Fig. 2b). Since the sequence-derived molecular mass of non-glycosylated human IFN- γ is 17.1 kDa (Gray *et al.*, 1982), this result suggested that the IFN- γ molecule had been proteolytically cleaved at two separate sites.

To confirm that all three bands represented non-glycosylated

Fig. 2. Proteolytic cleavage of IFN- γ in (a) control, (b) *N*-glycanase- and (c) tunicamycin-treated samples

Densitometry scans are shown of samples treated as in Fig. 1, lanes 2, 3 and 4. Values against peaks are molecular masses in kDa.

IFN- γ , cells were treated with tunicamycin to prevent the transfer of *N*-acetylglucosamine 1-phosphate to the dolichyl phosphate lipid carrier. As predicted, the same three bands were seen after this treatment (Fig. 1, lane 4, and Fig. 2c) as were found after denaturation of the glycoprotein followed by enzyme deglycosylation (Fig. 2b), except for a small fourth band with an intermediate molecular mass of 16 kDa. The three major non-glycosylated bands were also seen as a component of the control-treated and non-treated controls (Fig. 1, lanes 2 and 7 respectively). Together these results suggest that: (a) the IFN- γ variants with molecular masses of 19–27 kDa were all glycosylated; (b) some significant proteolytic cleavage of the amino acid backbone was occurring; and (c) the CHO line was secreting some non-glycosylated IFN- γ .

To discover whether the glycosylation heterogeneity observed was due to a variation in the terminal sialic acid content, samples were treated with neuraminidase to remove these residues (Fig. 1, lane 6), and control samples were mock-treated in the absence of enzyme (Fig. 1, lane 5). Neuraminidase caused the top cluster of six bands (23–27 kDa) to migrate further, representing an apparent 2 kDa decrease in molecular mass, whereas the three glycosylated bands of lower molecular mass were decreased by 0.5 kDa only.

Analysis of proteolysis

Since we found evidence for at least two truncated forms of IFN- γ (Fig. 2b and 2c), we looked for proteinase activity both within the cell and in the culture supernatant. In initial experiments, medium was harvested from CHO cells cultured for 72 h and filter-sterilized to ensure complete cell removal. Samples (1 ml) were incubated with 35 S-labelled IFN- γ for 0, 24, 48 and 96 h at 37 °C in the presence or absence of proteinase inhibitors. After immunoprecipitation, autoradiography and integration of the bands, we found no increase in the relative proportions of any of the IFN- γ variants, irrespective of the presence of proteinase inhibitors (results not shown). To investigate whether there was intracellular cleavage, pulse-chase analysis was used to monitor the kinetics of proteolysis in both cell lysates and secreted IFN- γ . Cells were pulsed with [35 S]methionine for 90 min and chased for 0, 30, 60 or 120 min with excess unlabelled methionine, with or without proteinase inhibitors (Figs. 3a and 3b). The percentage of IFN- γ which had been proteolytically cleaved within the cell at each time point was greater than that seen in the secreted IFN- γ , but there was little change in these proportions with time. The position of the cleaved forms of IFN- γ were confirmed by immunoprecipitation with monoclonal anti-peptide antibodies raised to the terminal regions of the IFN- γ

Fig. 3. Pulse-chase analysis of proteolytic cleavage of intracellular and extracellular IFN- γ

CHO cells were pulsed with [35 S]methionine for 90 min and then chased with medium containing excess non-labelled methionine for 30, 60 or 120 min. Both cell lysates (●) and supernatants (■) were immunoprecipitated, resolved on 14% gels and autoradiographed before scanning by densitometry. This Figure shows the percentage of cleaved IFN- γ present in (a) the fully-glycosylated IFN- γ and (b) the IFN- γ singly glycosylated forms.

Fig. 4. IFN- γ heterogeneity in batch cultures of CHO cells

(a) Starting concentration of 10 mM-glucose. Daily samples from a 2-litre CHO-cell stirred-fermenter culture were immunoprecipitated, resolved on 14% gels and silver-stained. The gels were then scanned and the peaks integrated to allow the proportion of each major glycosylation variant in the sample to be estimated on the basis of their respective molecular-mass values. Symbols: ○, glucose concentration (mM); ●, IFN- γ with two sites glycosylated; ■, one site glycosylated; ◆, no sites filled. (b) Starting concentration of 20 mM-glucose. Daily samples from a 2-litre CHO-cell culture were analysed as described for (a).

molecule (results not shown). Again, the presence of a mixture of proteinase inhibitors in the culture medium had no effect on these results (results not shown).

Glycosylation patterns in fermenter culture

The effects of extended batch culture on the pattern of IFN- γ secretion was investigated by harvesting daily samples from 2-litre fermenter cultures of CHO cells. Samples (1 ml) were centrifuged to remove cells, immunoprecipitated, and analysed by densitometry of silver-stained SDS/14%-PAGE gels. In the first experiments two fermenters were run in parallel for 142 h and showed similar results (one set of which is shown in Fig. 4a). Samples from both batch cultures showed a marked shift in the proportion of non-glycosylated IFN- γ as a function of time; after 141 h up to 25% of the IFN- γ was non-glycosylated. The percentage of fully glycosylated IFN- γ was correspondingly reduced. The limits of detection by silver-staining [approx. 3000 units (60 ng) of IFN- γ /track] prevented analysis during the first 48 h of these cultures, but it is known from previous experiments that, after 3 h of labelling, only 5–7% of the IFN- γ secreted is non-glycosylated (see Figs. 1 and 2a).

Glucose was almost depleted in these cultures after 118 h (Fig. 4a), which may have compromised the efficiency of glycosylation. To determine whether these glycosylation differences were influenced by glucose depletion, a 2-litre fermenter culture was initiated using double the normal concentration of glucose (20 mM; Fig. 4b). Although the glucose concentration never fell below 6 mM, a similar shift in the glycosylation pattern was seen, except that the proportion of singly glycosylated IFN- γ was 10% less throughout this culture (Fig. 4b). A subsequent fermenter culture was maintained for 195 h in order to monitor the changes in the proportion of non-glycosylated IFN- γ secreted over a longer time period, again using a starting concentration of 10 mM-glucose (Fig. 5). The proportion of the IFN- γ that was fully glycosylated gradually declined as the non-glycosylated form increased, from undetectable levels at 40 h to 30% of total IFN- γ at 158 h, before decreasing again to 20% of the total at 195 h. The specific growth rate and interferon production rate began to decline 50 h into the culture (Hayter *et al.*, 1990), which coincided with the first detectable appearance of non-glycosylated

Fig. 5. Extended batch culture of CHO cells using 10 mM-starting glucose concentration

Daily samples from a 2-litre CHO-cell culture were analysed as described for Fig. 4(a).

IFN- γ . The percentage of non-viable cells increased rapidly (from less than 10% of the total) after 166 h, coincident with a decrease in the relative amount of the non-glycosylated form.

Finally, we confirmed that the glycosylation shift was not an artefact caused by the fermenter design and operation, as daily samples from a 500 ml spinner culture of the same CHO-cell line exhibited a similar pattern of glycosylation, with the non-glycosylated IFN- γ increasing from 0% on day 2 to 30% by day 5. The fully glycosylated IFN- γ decreased from 60% to 30% over the same period (results not shown).

DISCUSSION

At least twelve molecular-mass variants of human IFN- γ were produced by the CHO-cell line transfected with the gene for IFN- γ . The variants were generated because of an inconsistency in the number of *N*-linked glycosylation sites occupied [no, one (Asn²⁸) or two (Asn²⁸ and Asn¹⁰⁰) sites filled] and as a result of differential proteolytic cleavage of the core polypeptide at two separate sites. In addition, there may have been some oligosaccharide heterogeneity at one position in the fully glycosylated form (Bulleid *et al.*, 1990), since six variants of this completely glycosylated form were found compared with three of the singly glycosylated form. Neuraminidase treatment diminished the molecular mass of each IFN- γ variant in a stepwise manner, indicating an invariant content of the sialic acid that terminates each oligosaccharide chain. The IFN- γ molecular-mass values observed here included those reported for the two glycosylated forms normally seen in natural IFN- γ (Rinderknecht *et al.*, 1984) and that produced by CHO cells (Mutsaers *et al.*, 1986).

Using tunicamycin and *N*-glycanase treatment we have found that most of the secreted IFN- γ polypeptide is truncated, owing to cleavage at two positions. The cleavage sites are likely to be at the *C*-terminal end of the 17.1 kDa mature polypeptide, since the *N*-terminus of IFN- γ may be an *N*-blocked glutamine residue (Gribaudo *et al.*, 1985). Previous analysis of CHO-derived IFN- γ by m.s. (Morris & Greer, 1988) revealed the presence of a major cleaved 15.2 kDa form which corresponds to the lowest-molecular-mass band seen in the data presented here (Figs. 1 and 2). The removal of 15 amino acid residues at the *C*-terminus was responsible for this decrease in molecular mass (Morris & Greer, 1988) and coincides with a cluster of four basic residues

representing a trypsin-like cleavage site. Honda *et al.* (1987) have also reported that an *E. coli*-derived IFN- γ was found with the four amino acids closest to the C-terminus absent; the product was a 16.6 kDa IFN- γ polypeptide. An IFN- γ variant of this size was also observed in the present study (Figs. 1 and 2).

Significant proteolysis occurred before secretion (Fig. 3), possibly after entry of the nascent glycosylated IFN- γ into the Golgi apparatus, since we have other evidence which suggests that cleavage does not occur in the endoplasmic reticulum (Bulleid *et al.*, 1990). By the use of anti-(N-terminus) and anti-(C-terminus) monoclonal antibodies (Ichimori *et al.*, 1987) it should be possible to define precisely the positions where proteolytic cleavage occurs and whether the two major glycosylated forms of IFN- γ are equally affected.

A truncated variant of murine IFN- γ has also been reported by Dijkmans *et al.* (1987), in a transfected CHO-cell line, that did not increase over 24 h in culture. These data support our evidence that proteolytic cleavage does not significantly increase with time in culture up to 166 h. However, as non-viable cells accumulated towards the end of batch culture, the proteolysis of non-glycosylated IFN- γ increased, possibly owing to the liberation of intracellular proteases (Fig. 5). Glycosylated forms of IFN- γ appeared to be more resistant to proteolysis, as has been observed for other proteins (reviewed by Elbein, 1987).

A reproducible shift in the overall IFN- γ glycosylation was seen in batch culture (Figs. 4 and 5) that was not seen when exogenous labelled IFN- γ was incubated for 96 h in the presence of cell-free conditioned medium. It was concluded that the absence of oligosaccharides on some IFN- γ forms was due to a change in the specific growth and metabolic rate of the cells, rather than an instability of IFN- γ carbohydrate residues in the culture medium. Hakimi & Atkinson (1980) found that rapidly growing virally infected fibroblasts produced glycopeptides with larger oligosaccharide side chains when compared with non-growing cells, suggesting that cell growth rate can affect glycosylation in mammalian cells. The mechanisms which underlie this reduction in glycosylation require further investigation.

Many recombinant products, such as erythropoietin (Kagawa *et al.*, 1988) and human chorionic gonadotropin (Matzuk & Boime, 1988), are now produced in CHO cells, and correct glycosylation is an important requisite for their activity and half-life *in vivo*. Furthermore, the position of vacant glycosylation sites on erythropoietin can affect rates of biosynthesis and secretion (Dube *et al.*, 1988). In the present study we found that one of the two possible glycosylation sites on recombinant human IFN- γ is more likely to be vacant with increasing time in batch culture. The degree of glycosylation of other recombinant proteins therefore needs to be analysed during batch culture to discover whether the results presented here are typical of recombinant proteins expressed in CHO cells.

We thank Richard Willson and Adrian Whitfield for expert technical assistance, Wellcome Biotechnology Ltd. for the CHO-cell line, Celltech Ltd. for monoclonal antibody 20D7 and the SERC (Science and Engineering Research Council) Biotechnology Directorate, Celltech, Glaxo, Porton International, Smith-Kline Beecham and Wellcome

Biotechnology for support of the research programme of which the work described here was a part.

REFERENCES

- Arakawa, T., Hsu, Y. R., Parker, C. G. & Lai, P. H. (1986) *J. Biol. Chem.* **261**, 8534–8539.
- Ashwell, G. & Hartford, J. (1982) *Annu. Rev. Biochem.* **51**, 531–554.
- Bulleid, N., Curling, E. M. A., Freedman, R. B. & Jenkins, N. (1990) *Biochem. J.* **268**, 777–781.
- De La Maza, L., Peterson, E. M., Burton, L. E., Gray, P. W., Rinderknecht, E. & Czarniecki, C. W. (1987) *Infect. Immun.* **55**, 2727–2733.
- Elbein, A. D. (1987) *Annu. Rev. Biochem.* **56**, 497–534.
- Dijkmans, R., Heremans, H. & Billiau, A. (1987) *J. Biol. Chem.* **262**, 2528–2535.
- Dube, S., Fisher, J. W. & Powell, J. S. (1988) *J. Biol. Chem.* **263**, 17516–17521.
- Gray, P. W., Leung, D. W., Pennica, D., Yelverton, E., Najarian, R., Simonsen, C. C., Derynck, R., Sherwood, P. J., Wallace, D. M., Berger, S. L., Levinson, A. D. & Goeddel, D. V. (1982) *Nature (London)* **295**, 503–508.
- Gribaudo, G., Cofano, F., Prat, M., Baiocchi, C., Cavallo, G. & Landolfo, S. (1985) *J. Biol. Chem.* **260**, 9936–9940.
- Hakimi, J. & Atkinson, P. H. (1980) *Biochemistry* **19**, 5619–5624.
- Hayter, P., Curling, E., Baines, A., Bull, A., Salmon, I., Strange, P. & Jenkins, N. (1990) in *Production of Biologicals from Animal Cells in Culture* (ESACT Meet. 10th), Butterworths, in the press.
- Honda, S., Asano, T., Kajio, T., Nakagawa, S., Ikeyama, S., Ichimori, Y., Sugino, H., Nara, K., Kakinuma, A. & Kung, H. F. (1987) *J. Interferon Res.* **7**, 145–154.
- Hogrefe, H. H., McPhie, P., Bekisz, J. B., Enterline, J. C., Dyer, D., Webb, D. S. A., Gerrard, T. L. & Zoon, K. C. (1989) *J. Biol. Chem.* **264**, 12179–12186.
- Hubbard, S. C. & Ivatt, R. J. (1981) *Annu. Rev. Biochem.* **50**, 555–583.
- Ichimori, Y., Suzuki, N., Kitada, C. & Tsukamoto, K. (1987) *Hybridoma* **6**, 173–181.
- Johnson, A. & Thorpe, R. (1987) *Immunochemistry in Practice*, 2nd edn., pp. 150–151, Blackwells, Oxford.
- Kagawa, Y., Takasaki, S., Utsumi, J., Hosoi, K., Shimizu, H., Kochibe, N. & Kobata, A. (1988) *J. Biol. Chem.* **263**, 17508–17515.
- Kelker, H. C., Yip, Y. K., Anderson, P. & Vilcek, J. (1983) *J. Biol. Chem.* **258**, 8010–8013.
- Konrad, M. (1989) *Trends Biotechnol.* **7**, 175–179.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Matzuk, M. M. & Boime, I. (1988) *J. Cell Biol.* **106**, 1049–1059.
- McCormick, F., Trahey, M., Innis, M., Dieckmann, B. & Ringold, G. (1984) *Mol. Cell. Biol.* **4**, 166–172.
- Morris, H. R. & Greer, F. M. (1988) *Trends Biotechnol.* **6**, 140–147.
- Mutsaers, J. H. G. M., Kamerling, J. P., Devos, R., Guisez, Y., Friers, W. & Vliegthart, J. F. G. (1986) *Eur. J. Biochem.* **156**, 651–654.
- Rinderknecht, E., O'Connor, B. H. & Rodriguez, H. (1984) *J. Biol. Chem.* **259**, 6790–6797.
- Sakaguchi, M., Honda, S., Ozawa, M. & Nishimura, O. (1988) *FEBS Lett.* **230**, 201–204.
- Seelig, G. F., Wijdenes, J., Nagabhashan, T. L. & Trotta, P. P. (1988) *Biochemistry* **27**, 1981–1987.
- Steis, R. G., Smith, J. W., Urba, W. J., Clark, J. W., Itri, L. M., Evans, L. M., Schoenberger, C. & Longo, D. L. (1988) *N. Engl. J. Med.* **318**, 1409–1413.
- Trinchieri, G. & Perussia, B. (1985) *Immunol. Today* **6**, 131–136.
- Utsumi, J., Mizuno, Y., Hosoi, K., Okano, K., Sawada, R., Kajitani, M., Sakai, I., Naruto, M. & Shimizu, H. (1989) *Eur. J. Biochem.* **181**, 545–553.